DESCRIPTION

NOVEL CAROTENOID HYDROXYLASE GENE, METHOD FOR PREPARING HYDROXYLATED CAROTENOID, AND NOVEL GERANYLGERANYL PYROPHOSPHATE SYNTHASE

TECHNICAL FIELD

The present invention relates to a novel enzyme that introduces a hydroxyl group at the position 2 (2') carbon of the β-ionone ring in carotenoids, a gene encoding the enzyme, and a microorganism into which the gene is introduced. The present invention also relates to a method of preparing a carotenoid in which the position 2 carbon of its β-ionone ring is hydroxylated, using the above microorganism.

Further, the present invention relates to a gene encoding a novel enzyme that introduces a hydroxyl group at the position 3 (3') carbon of the β-ionone ring in carotenoids, and a microorganism into which the gene is introduced. The present invention also relates to a method of preparing a carotenoid in which the position 3 carbon of its β-ionone ring is hydroxylated, using the above microorganism.

Further, the present invention relates to a gene encoding a novel geranylgeranyl pyrophosphate (GGPP) synthase.

BACKGROUND ART

Carotenoid (also called "carotinoid") is a general term for pigments occurring abundantly in nature, which are built up from an isoprene backbone of 40 carbon atoms. To date, more than 700 species of carotenoids have been isolated (Britton, G., Liaaen-Jensen, S., and Pfander, H., Carotenoids Handbook, Birkhauser Verlag, Basel, 2004). Recently, prophylactic effects of carotenoids on chronic diseases such as cancer have attracted people's attention, and a great number of reports have been made (see, for example, H. Nishino, M. Murakoshi and M. Yano, Food Style 21, 4, 53-55, 2000; Nishino, H. et al, "Carotenoids in cancer chemoprevention", Cancer Metastasis Rev. 21, 257-264, 2002; Mayne, S.T., "β-Carotene, carotenoids, and disease prevention in humans", FASEB J., 10, 690-701, 1996).

Although carotenoids have a huge variety of species, species which have been

used in prophylactic studies (human epidemiological/clinical tests, animal administration tests, etc.) are extremely limited. Those carotenoids include β-carotene (also called β-carotine; chemically synthesized product), lycopene (also called lycopine; extracted from tomato), α-carotene (also called α-carotine; extracted from palm oil), lutein (extracted from marigold), astaxanthin (extracted from krill or *Haematococcus* alga, or chemically synthesized), fucoxanthin (extracted from edible marine algae) and β-cryptoxanthin (extracted from mandarin orange). The results of cancer prophylactic studies using these pigments gradually revealed that cancer prophylactic effects of carotenoids vary depending on the species of carotenoids. As an example, the results of experiments using mice conducted by Nobuo Takasuka et al. of the Research Institute, National Cancer Center (Report of 1996 Meeting on Carotenoid Research) will be shown below. The incidence of lung cancer (ddy mouse lung two-stage carcinogenesis model) was 40% in α-carotene-administered mice, 70% in lutein- or astaxanthin-administered mice and 139% in β-carotene-administered mice, when the incidence in control mice without carotenoid administration was taken as 100%. The incidence of liver cancer (mouse spontaneous liver carcinogenesis model) was 30% in astaxanthin- or fucoxanthin-administered mice, 50 % in α-carotene- or lutein-administered mice, 70% in β-carotene-administered mice, and 100% in lycopene-administered mice, when the incidence in control mice without carotenoid administration was taken as 100%. The incidence of skin cancer (mouse skin carcinogenesis model) was 10% in fucoxanthin- or lycopene-administered mice, and 100% in astaxanthin-administered mice, when the incidence in control mice without carotenoid administration was taken as 100%. Comparison of the results on these three carcinogenesis models reveals that lycopene which was highly effective in inhibiting lung cancer and skin cancer is not effective in inhibiting liver cancer; and that astaxanthin which was highly effective in inhibiting liver cancer is not effective in inhibiting skin cancer. Further, as a result of epidemiological tests and clinical tests, it has been reported that, among dietary carotenoids, only lycopene was confirmed as a prophylactic carotenoid against prostate cancer (see Giovannucci, E., Ascherio, A., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Willet, W. C., "Intake of carotenids and retinol in relation to risk of prostate cancer", J. National Cancer Institute 87, 1767-1776, 1995; Vogt, T.M. et al, "Serum lycopene, other serum carotenoids, and risk of prostate cancer in US Blacks and Whites", Am. J. Epidemiol. 155, 1023-1032, 2002). Recently, high prophylactic effect of

β-cryptoxanthin on lung cancer has been gradually elucidated (see Yuan, J.M., Stram, D.O., Arakawa, K., Lee, H.P. and Yu M.C., "Dietary cryptoxanthin and reduced risk of lung cancer: the Singapore Chinese Health Study", Cancer Epidemiol. Biomarkers Prev. 12, 890-898, 2003; Mannisto, S. et al, "Dietary carotenoids and risk of lung cancer in a pooled analysis of seven cohort studies", Cancer Epidemiol. Biomarkers Prev. 13, 40-48, 2004). In addition to cancer prophylactic effects, it has been also reported that carotenoids are very likely to be effective in preventing chronic diseases in the cardiovascular system, chronic diseases in the eye such as cataract, and chronic diseases such as osteoporosis. For example, it has been reported that carotenoids which are expected to be effective on chronic diseases in the eye (age-related macular degeneration, cataract, etc.) are lutein and zeaxanthin alone among dietary carotenoids (see Semba, R.D. and Dagnelie, G., "Are lutein and zeaxanthin conditionally essential nutrients for eye health?", Med. Hypotheses 61, 465-472, 2003; Mazaffarieh, M., Sacu, S. and Wedrich, A., "The role of the carotenoids, lutein and zeaxanthin, in protecting against age-related macular degeneration: A review based on controversial evidence", Nutr. J., 2, 20, 2003).

The results described so far indicate that only about 10 out of 700 or more species of carotenoids have been examined for their prophylactic effects on chronic diseases such as cancer in studies actually using animal individuals, and that each carotenoid has characteristic individuality in prophylactic effect on chronic diseases such as cancer. It is believed that the major reason why the number of species of carotenoids actually examined is so mall is because those carotenoids which can be extracted, purified or chemically synthesized in large quantities are limited to the above-mentioned carotenoids.

As a promising means to solve the above problem, a method may be considered in which a carotenoid of interest is mass-produced in carotenoid biosynthesis gene-transferred yeast or *Escherichia coli*. For example, Shimada et al. of Kirin Brewery introduced a carotenoid biosynthesis gene cluster into a food yeast *Candida utilis* which naturally does not biosynthesize carotenoids, expressed the genes and succeeded in synthesizing 7.8 mg/g of lycopene (dry weight) (Shimada, H., Kondo, K., Fraser, P. D., Miura, Y., Saito, T., and Misawa, N., "Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway", Appl. Environ. Microbiol., 64, 2676-2680, 1998). According to the gene recombination technique, it becomes possible to produce such carotenoids that have not been found in nature or found

only in trace amounts, by a combination of various biosynthesis genes. For example, Takaichi et al. of Nippon Medical School have succeeded in producing parasiloxanthin (which is only reported to be found in catfish in a trace amount) as a major carotenoid product in a recombinant E. coli (Takaichi, S., Sandmann, G., Schnurr, G., Satomi, Y., Suzuki, A., and Misawa, N. "The carotenoid 7,8-dihydro-Ψ end group can be cyclized by the lycopene cyclases from the bacterium *Erwinia uredovora* and the higher plant *Capsicum annuum*", Eur. J. Biochem., 241, 291-296, 1996). There is another report that astaxanthin-β-diglucoside, a "non-natural type" carotenoid not found in nature, was produced in a recombinant *E. coli* (Yokoyama, A., Shizuri, Y.,and Misawa, N., Production of new carotenoids, astaxanthin glucosides, by *Escherichia coli* transformants carrying carotenoid biosynthetic genes. Tetrahed. Lett., 39, 3709-3712, 1998).

The carotenoid biosynthesis genes most commonly used in the preparation of recombinant microorganisms for various carotenoid productions are derived from *Erwinia* bacteria (such as *Erwinia uredovora*; recently, this bacterium is called *Pantoea ananatis*). Six genes have been isolated from *Erwinia* bacteria; they are *crtE*, *crtB*, *crtI*, *crtY*, *crtZ* and *crtX*. The functions of the biosynthesis enzymes encoded by these genes (CrtE, CrtB CrtI, CrtY, CrtZ and CrtX) are shown in Fig. 1 (see Non-Patent Document 1). When biosynthesis of astaxanthin is intended, *crtW* gene derived from marine bacteria *Paracoccus* [such as *Paracoccus* sp. MBIC 01143 (*Agrobacterium aurantiacum*)] is additionally required (Fig. 1). Five gene have been isolated from *Paracoccus* bacteria; they are crtB, *crtI*, *crtY*, *crtZ* and *crtW* (see Non-Patent Document 1). The functions of *crtB*, *crtI*, *crtY* and *crtZ* genes are common in both bacteria. When *Erwinia*- or *Paracoccus*-derived *crtE*, *crtB*, *crtI* and *crtY* genes have been introduced and expressed in *E. coli*, the *E. coli* biosynthesizes β-carotene. When marine bacterium-derived *crtW* gene and *Erwinia*- or *Paracoccus*-derived *crtZ* gene are further introduced and expressed in the above *E. coli*, the recombinant *E. coli* begins to synthesize astaxanthin. Further, when *Erwinia*-derived *crtX* gene is introduced and expressed in this *E. coli* synthesizing astaxanthin, the recombinant *E. coli* begins to synthesize a "non-natural type" carotenoid, astaxanthin-β-diglucoside (Fig. 1).

As described so far, it is being demonstrated that carotenoid biosynthesis genes can be used to produce "rare" carotenoids which occur only in trace amounts in nature or "non-natural type" carotenoids existence of which has not been confirmed. On the other

hand, the carotenoid biosynthesis genes which may be used for this purpose are limited to 25 genes. They are *crtM* (dehydrosqualene synthase), *crtE* (*gps*, *al-3*) (geranylgeranyl pyrophosphate synthase), *crtB* (*psy*, *al-2*) (phytoene synthase), *crtN* (dehydrosqualene desaturase), *crtP* (*pds1*) (phytoene desaturase: addition of two double bonds), *crtQ* (*zds*) (ζ-carotene desaturase: addition of two double bonds), *crtI* (derived from *Rhodobacter*) (phytoene desaturase: addition of three double bonds and cis-trans isomerization), *crtI* (phytoene desaturase: addition of four double bonds and cis-trans isomerization), *al-1* (phytoene desaturase: addition of five double bonds and cis-trans isomerization), *crtY* (*crtL*-β) (lycopene β-cyclase), *crtL*-ε (lycopene ε-cyclase), *crtYm* (lycopene β-monocyclase), *crtU* (β-carotene desaturase), *crtZ* (β-carotene hydroxylase; β-C3-hydroxylase), *crtW* (*bkt*) (β-carotene ketolase; β-C4-oxygenase), *crtO* (derived from *Synechocystis* sp. PCC6803) (β-carotene monoketolase), *crtX* (zeaxanthin glucosyltransferase), *crtC* (hydroxyneurosporene synthase), *crtD* (methoxyneurosporene desaturase), *crtF* (hydroxyneurosporene o-methyltransferase), *crtA* (spheroidene monooxygenase), *crtEb* (lycopene elongase), *crtYe/Yf* (decaprenoxanthin synthase), *zep1* (zeaxanthin epoxydase) and *ccs* (capsanthin/capsorubin synthase) (see Lee, P.C. and Schmidt-Dannert, C., "Metabolic engineering towards biotechnological production of carotenoids in microorganisms", Appl. Microbiol. Biotechnol. 60, 1-11, 2002; Teramoto, M., Takaichi, S., Inomata, Y., Ikenaga, H. and Misawa, N. "Structural and functional analysis of a lycopene β-monocyclase gene isolated from a unique marine bacterium that produces myxol", FEBS Lett. 545, 120-126, 2003). In order to allow microorganisms such as *E. coli* to produce a wide variety of carotenoids, novel carotenoid biosynthesis genes must be isolated. However, cloning of novel carotenoid biosynthesis genes makes very slow progress. For example, while carotenoids occurring most abundantly in nature are those with β-ionone rings (in Fig. 1, β-carotene, zeaxanthin, canthaxanthin, astaxanthin, etc.), only two genes of enzymes which hydroxylate or oxygenate β-ionone rings have been isolated. They are genes encoding β-ionone ring-3-hydroxylase (β-C3-hydroxylase) (CrtZ) and β-ionone ring-4-ketolase (β-C4-ketolase; β-C4-oxygenase) (CrtW), respectively. These enzyme genes were isolated as early as in 1990 for *crtZ* and in 1995 for *crtW*, and analyzed for their functions. It is believed that a gene of β-ionone ring-2-hydroxylase is necessary for synthesizing carotenoids such as nostoxanthin in which the position 2 carbon of the β-ionone ring is hydroxylated. However, though there are some microorganisms

producing such carotenoids (see Non-Patent Document 2), nothing has been found to date about such an enzyme or gene. It seems that the reason why the cloning of novel carotenoid biosynthesis genes is difficult is because those carotenoid biosynthesis genes obtainable by expression cloning in *E. coli* or by cloning using homology to existing carotenoid genes have already been obtained and all the remaining genes are not obtainable by these cloning methods.

Carotenoids consisting of carbon and hydrogen alone are called carotene, and carotenoids comprising oxygen-containing functional groups, such as hydroxyl group or keto group, in addition to carbon and hydrogen are called xanthophyll. Carotene and xanthophyll are greatly different in physical property and considerably different in physiological activity in the living body. For example, β-cryptoxanthin is a carotenoid in which one hydroxyl group is introduced at the position 3 carbon of β-carotene. It is known that the intake ratio of β-cryptoxanthin into the living body is 10 times higher than that ratio of β-carotene. β-Cryptoxanthin is a carotenoid which has been especially attracting attention in Japan recently. Data showing its prophylactic effects on large bowel cancer, cervix cancer, esophageal cancer, prostate cancer, rheumatoid and osteoporosis in addition to the above-described lung cancer are being gathered (Yano, M., Report of 2003 Meeting on Carotenoid Research and the above-mentioned Yuan, J.M., Stram, D.O., Arakawa, K., Lee, H.P. and Yu M.C., Cancer Epidemiol. Biomarkers Prev. 12, 890-898, 2003 and Mannisto, S. et al, Cancer Epidemiol. Biomarkers Prev. 13, 40-48, 2004). Such effects are not recognized in β-carotene. A carotenoid in which two hydroxyl groups are introduced at both positions 3 and 3' of β-carotene is zeaxanthin (see Fig. 1). As described above partially, it is known that the physiological activity of zeaxanthin is different from that of β-cryptoxanthin. A carotenoid in which the both methylene groups at positions 4 and 4' of zeaxanthin are converted to keto groups is astaxanthin (see Fig. 1). As described above partially, the physiological activity of astaxanthin in cancer prevention is also greatly different from that of β-carotene. On the other hand, a carotenoid in which two hydroxyl groups are further introduced at both positions 2 and 2' of zeaxanthin is nostoxanthin. Generally, carotenoids such as nostoxanthin in which two hydroxyl groups are introduced at positions 2 and 2' of β-ionone rings occur only in trace amounts in nature, and it is impossible to produce them in large quantities. Thus, prophylactic studies against various chronic diseases such as

cancer cannot be conducted. The enzyme that introduces hydroxyl groups at positions 3 and 3' of β-ionone rings is CrtZ. However, no CrtZ proteins having a 49% or less identity with the *Erwinia uredovora*-derived CrtZ reported in a paper in 1990 for the first time (Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K., and Harashima, K., *J. Bacteriol.* 172, 6704-6712, 1990) have been isolated to date. Among the CrtZ proteins which have been confirmed to have the same function, the enzyme that has the highest homology to the *Erwinia uredovora*-derived CrtZ is a *Paracoccus zeaxanthinifaciens* (old designation: *Flavobacterium* sp. R1534)-derived CrtZ (Pasamontes, L., Hug, D., Tessier, M., Hohmann, H. P., Schierle, J., and van Loon, A. P., *Gene* 185, 35-41, 1997) with a 50% identity.

(Non-Patent Document 1) Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Saito, T., Ohtani, T., and Miki, W., "Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level", J. Bacteriol., 177, 6575-6584, 1995)

(Non-Patent Document 2) Yokoyama, A., Miki, W., Izumida, H., and Shizuri, Y., "New trihydroxy-keto-carotenoids isolated from an astaxanthin-producing marine bacterium", Biosci. Biotech. Bioche., 60, 200-203, 1996)

DISCLOSURE OF THE INVENTION

Problem for Solution by the Invention

It is an object of the present invention to isolate a gene encoding an enzyme that hydroxylates position 2 of β-ionone ring (β-ionone ring-2-hydroxylase). Further, the present invention aims at providing a method of producing carotenoids in which position 2 of the β-ionone ring is hydroxylated (such as 2-hydroxyastaxanthin or nostoxanthin), using a recombinant microorganism in which the above gene is introduced and expressed.

It is another object of the present invention to isolate a gene encoding a carotenoid biosynthesis related enzyme (such as enzyme that hydroxylates position 3 of β-ionone ring; β-ionone ring-3-hydroxylase) with a low homology to existing genes. Further, the present invention aims at providing a method of producing carotenoids in which position 3 of the β-ionone ring is hydroxylated (such as astaxanthin or zeaxanthin), using a recombinant microorganism in which the above gene is introduced and expressed.

Means to Solve the Problem

The present inventors paid attention to the fact that a marine bacterium *Brevundimonas* sp. strain SD-212 (MBIC 03018) is capable of producing carotenoids such as 2-hydroxyastaxanthin and 2-hydroxyadonixanthin in which position 2 of the β-ionone ring is hydroxylated. As a result of intensive and extensive researches, the present inventors have succeeded for the first time in the world in isolating a gene encoding an enzyme that hydroxylates position 2 of the β-ionone ring (β-ionone ring-2-hydroxylase). Among the carotenoid biosynthesis gene cluster encoding the above-mentioned enzyme, the present inventors have found genes encoding an enzyme that hydroxylate position 3 of the β-ionone ring (β-ionone ring-3-hydroxylase) and a geranylgeranyl pyrophosphate (GGPP) synthase, with extremely low homologies to existing genes. The catalytic functions of these enzymes could be confirmed.

First, a cosmid library was prepared in *E. coli* using the chromosomal DNA of *Brevundimonas* sp. strain SD-212. In the preparation of a cosmid library in *E. coli* using the chromosomal DNA of a carotenoid-producing *Erwinia* bacterium (such as *Erwinia uredovora*), yellow colonies (carotenoid-producing *E. coli*) are obtained on plates at this stage. Therefore, a carotenoid biosynthesis gene cluster can be obtained easily. However, no *E. coli* colonies showing color change were obtained from the cosmid library of *Brevundimonas* sp. strain SD-212. Then, a cosmid library of *Brevundimonas* sp. strain SD-212 was prepared in a recombinant *E. coli* (yellow colonies) which produces zeaxanthin as a result of introduction of *Erwinia uredovora*-derived *crtE*, *crtB*, *crtI*, *crtY* and *crtZ* genes (see Fig. 1). In the resultant cosmid library, no colonies showing color change were obtained. Then, 700 colonies were cultured and subjected to examination with HPLC-PDA (photodiode detector) to see whether a novel carotenoid is produced in addition to the control (zeaxanthin). As a result, no colonies produced a novel carotenoid. Therefore, the inventors concluded that it is impossible to conduct expression cloning of carotenoid biosynthesis genes of *Brevundimonas* sp. strain SD-212.

Subsequently, the present inventors have found that phytoene desaturase (*crtI*) genes have two conserved domains among carotenoid-producing bacteria and designed PCR primers choosing these domains. Using the resultant primers, PCR was performed with the chromosomal DNA of *Brevundimonas* sp. strain SD-212 as a template. As a result, a 1.1 kb DNA fragment was amplified. The nucleotide sequence of this fragment was determined and found to be a partial sequence of *crtI*. Colony hybridization of strain

SD-212 cosmid library was performed with the partial sequence of *crtI* as a probe. Several positive colonies were obtained. Plasmid DNA was prepared from the positive colonies and subjected to Southern hybridization, to thereby obtain a positive 12 kb *Eco*RI DNA fragment. The nucleotide sequence of this 12 kb *Eco*RI fragment was determined. Fortunately, it has become clear that a carotenoid biosynthesis gene cluster [seven open reading frames (ORFs) having homology to existing six *crt* genes and one *idi* gene] is present within this fragment. Further, it was found that five unknown ORFs are present within this 12 kb *Eco*RI fragment. For forced expression of all of these 12 ORFs in *E. coli* by the fusion protein method using the *lac* promoter in *E. coli* vector pUC18 and a *lacZ* leader sequence, constructs were prepared. Then, using *Erwinia-* or *Paracoccus*-derived *crt* genes, functional analysis of these 12 ORFs was performed in *E. coli* cells producing various carotenoids as hosts. The results revealed that the six ORFs having homology to existing carotenoid biosynthesis (*crt*) genes are carotenoid biosynthesis (*crt*) genes having functions similar to those of the existing carotenoid biosynthesis (*crt*) genes. Surprisingly, however, it was found that *crtZ* gene which is one of these carotenoid biosynthesis genes encodes a β-ionone ring-3-hydroxylase having only a 46% or less identity with any of the existing CrtZ proteins at the amino acid sequence level. Further, it was also found that *crtE* gene which is one of these carotenoid biosynthesis genes encodes a geranylgeranyl pyrophosphate (GGPP) synthase having only a 39% or less identity with any of the existing CrtE proteins at the amino acid sequence level. Further, the present inventors have ascertained that one of the unknown ORFs (ORF11) is a gene encoding a β-ionone ring-2-hydroxylase. Thus, the present invention has been achieved.

The present invention has been achieved based on the above-described findings.

The present invention provides the following (1) to (17).

(1) A peptide selected from the following (a), (b), (c) or (d):

(a) a peptide consisting of the amino acid sequence as shown in SEQ ID NO: 4;

(b) a peptide which consists of the amino acid sequence as shown in SEQ ID NO: 4 having addition, deletion or substitution of one or a plurality of amino acids and has β-ionone ring-2-hydroxylase activity;

(c) a peptide which consists of an amino acid sequence having a 50% or more identity with the amino acid sequence as shown in SEQ ID NO: 4 and has β-ionone

ring-2-hydroxylase activity; or

(d) a bacterium-derived peptide which is encoded by a DNA consisting of the nucleotide sequence as shown in SEQ ID NO: 3 or a DNA hybridizable to a complementary DNA to the above DNA under stringent conditions and has β-ionone ring-2-hydroxylase activity.

(2) A gene encoding a peptide selected from the following (a), (b), (c) or (d):

(a) a peptide consisting of the amino acid sequence as shown in SEQ ID NO: 4;

(b) a peptide which consists of the amino acid sequence as shown in SEQ ID NO: 4 having addition, deletion or substitution of one or a plurality of amino acids and has β-ionone ring-2-hydroxylase activity;

(c) a peptide which consists of an amino acid sequence having a 50% or more identity with the amino acid sequence as shown in SEQ ID NO: 4 and has β-ionone ring-2-hydroxylase activity; or

(d) a bacterium-derived peptide which is encoded by a DNA consisting of the nucleotide sequence as shown in SEQ ID NO: 3 or a DNA hybridizable to a complementary DNA to the above DNA under stringent conditions and has β-ionone ring-2-hydroxylase activity.

(3) A microorganism obtainable by introducing the gene according to (2) thereinto, wherein the microorganism is capable of introducing a hydroxyl group at the position 2 carbon of β-ionone ring.

(4) A microorganism obtainable by introducing the gene according to (2) and other carotenoid biosynthesis genes thereinto, wherein the microorganism is capable of introducing a hydroxyl group at the position 2 carbon of β-ionone ring.

(5) The microorganism according to (4), wherein the other carotenoid biosynthesis genes are all or a part of a gene cluster required for synthesizing β-ionone ring-containing carotenoids from farnesyl pyrophosphate.

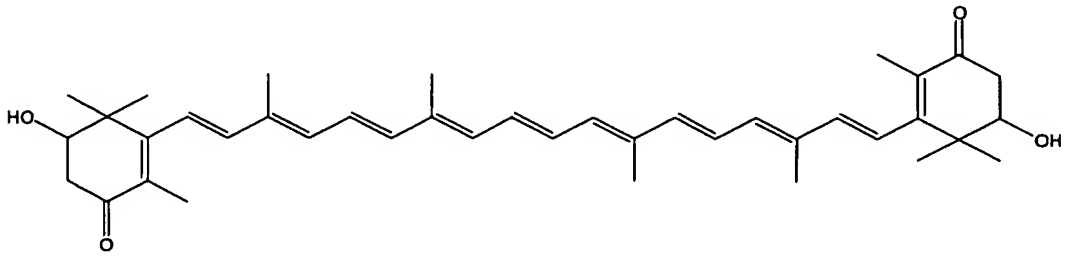
(6) The microorganism according to any one of (3) to (5), wherein the microorganism is *Escherichia coli*.

(7) A method of preparing a hydroxylated carotenoid, comprising culturing the microorganism according to any one of (3) to (6) in a medium and obtaining from the resultant culture or cells a carotenoid which is hydroxylated at the position 2 carbon of its β-ionone ring.

(8) The method according to (7), wherein the carotenoid which is hydroxylated at the position 2 carbon of its β-ionone ring is β,β-carotene-2-ol (2-hydroxy-β-carotene),

β,β-carotene-2,2'-diol (2,2'-dihydroxy-β-carotene), caloxanthin (2-hydroxyzeaxanthin), nostoxanthin (2,2'-dihydroxyzeaxanthin), 2-hydroxy-β,β-carotene-4,4'-dione (2-hydroxycanthaxanthin), 2,2'-dihydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxycanthaxanthin), 2-hydroxyastaxanthin or 2,3,2',3'-tetrahydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxyastaxanthin).

(9) 2,2'-dihydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxycanthaxanthin) represented by the following chemical formula (I):



(I)

(10) An antioxidant comprising 2,2'-dihydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxycanthaxanthin) or 2-hydroxy-β,β-carotene-4,4'-dione (2-hydroxycanthaxanthin) as an active ingredient.

(11) A gene encoding a peptide selected from the following (e), (f) or (g):

(e) a peptide consisting of the amino acid sequence as shown in SEQ ID NO: 30;

(f) a peptide which consists of the amino acid sequence as shown in SEQ ID NO: 30 having addition, deletion or substitution of one or a plurality of amino acids and has β-ionone ring-3-hydroxylase activity; or

(g) a bacterium-derived peptide which is encoded by a DNA consisting of the nucleotide sequence as shown in SEQ ID NO: 29 or a DNA hybridizable to a complementary DNA to the above DNA under stringent conditions and has β-ionone ring-3-hydroxylase activity.

(12) A microorganism obtainable by introducing the gene according to (11) thereinto, wherein the microorganism is capable of introducing a hydroxyl group at the position 3 carbon of β-ionone ring.

(13) A microorganism obtainable by introducing the gene according to (11) and other carotenoid biosynthesis genes thereinto, wherein the microorganism is capable of

introducing a hydroxyl group at the position 3 carbon of β-ionone ring.

(14) The microorganism according to (13), wherein the other carotenoid biosynthesis genes are all or a part of a gene cluster required for synthesizing β-ionone ring-containing carotenoids from farnesyl pyrophosphate.

(15) The microorganism according to any one of (12) to (14), wherein the microorganism is *Escherichia coli*.

(16) A method of preparing a hydroxylated carotenoid, comprising culturing the microorganism according to any one of (12) to (15) in a medium and obtaining from the resultant culture or cells a carotenoid which is hydroxylated at the position 3 carbon of its β-ionone ring.

(17) A gene encoding a peptide selected from the following (h), (i) or (j):

(h) a peptide consisting of the amino acid sequence as shown in SEQ ID NO: 32;

(i) a peptide which consists of the amino acid sequence as shown in SEQ ID NO: 32 having addition, deletion or substitution of one or a plurality of amino acids and has geranylgeranyl pyrophosphate synthase activity; or

(j) a bacterium-derived peptide which is encoded by a DNA consisting of the nucleotide sequence as shown in SEQ ID NO: 31 or a DNA hybridizable to a complementary DNA to the above DNA under stringent conditions and has geranylgeranyl pyrophosphate synthase activity.

Hereinbelow, the present invention will be described in detail.

1. Gene Source: Marine Bacterium *Brevundimonas* sp. strain SD-212 (MBIC 03018)

The marine bacterium *Brevundimonas* sp. strain SD-212 (SD212; MBIC 03018) which was the source for genes of interest is α-proteobacterium isolated from the seawater around Volcano Islands belonging to Ogasawara Islands. GC content is 67.1 (mol) %. Yokoyama et al. of Marine Biotechnology Institute Co., Ltd. have reported that carotenoids produced by this marine bacterium are 2(2')-hydroxylated carotenoids such as 2-hydroxyastaxanthin and 2-hydroxyadonixanthin (see Non-Patent Document 2). By the way, this bacterium is published and released by Marine Biotechnology Institute Co., Ltd. The 16S rDNA sequence and *gyrB* gene sequence of this bacterium are registered at GenBank/DDBJ under Accession Nos. AB016849 and AB014993, respectively.

2. Estimate of the Carotenoid Biosynthesis Pathway in the Marine Bacterium

Brevundimonas sp. strain SD-212

The 2(2')-hydroxylated carotenoids produced by the marine bacterium *Brevundimonas* sp. strain SD-212 (MBIC 03018) are analyzed in detail by Yokoyama et al. (see Non-Patent Document 2). They are 2,3,2',3'-tetrahydroxy-β,β-carotene-4,4'-dione, 2,3,2',3'-tetrahydroxy-β,β-carotene-4-one, 2-hydroxyastaxanthin (2,3, 3'-trihydroxy-β,β-carotene-4,4'-dione), 2-hydroxyadonixanthin (2,3, 3'-trihydroxy-β,β-carotene-4-one), and erythroxanthin (3,2',3'-trihydroxy-β,β-carotene-4-one) (see Fig. 2). It has been also confirmed that astaxanthin and adonixanthin (4-ketozeaxanthin) are also present in strain SD-212 as precursors. Given the existence of a novel enzyme that introduces a hydroxyl group at position 2 of the β-ionone ring (β-ionone ring-2-hydroxylase; designated "CrtV"), a biosynthesis pathway using this enzyme and a combination of existing Crt proteins, for all of the above carotenoids may be estimated as described in Fig. 2.

3. Gene Encoding β-Ionone Ring-2-Hydroxylase (The First Gene of the Invention)

The present invention includes the peptide of the following (a), (b), (c) or (d).

(a) a peptide consisting of the amino acid sequence as shown in SEQ ID NO: 4;

(b) a peptide which consists of the amino acid sequence as shown in SEQ ID NO: 4 having addition, deletion or substitution of one or a plurality of amino acids and has β-ionone ring-2-hydroxylase activity;

(c) a peptide which consists of an amino acid sequence having a 50% or more identity with the amino acid sequence as shown in SEQ ID NO: 4 and has β-ionone ring-2-hydroxylase activity; or

(d) a bacterium-derived peptide which is encoded by a DNA consisting of the nucleotide sequence as shown in SEQ ID NO: 3 or a DNA hybridizable to a complementary DNA to the above DNA under stringent conditions and has β-ionone ring-2-hydroxylase activity.

The peptide of (a) is a *Brevundimonas* sp. strain SD-212-derived peptide (sometimes called "CrtV") having β-ionone ring-2-hydroxylase activity and consisting of a 257 amino acid sequence.

The peptide of (b) is a peptide of (a) into which mutations that would not eliminate β-ionone ring-2-hydroxylase activity have been introduced. Such mutations include mutations occurring spontaneously in nature and artificial mutations. Examples of means to cause artificial mutations include, but are not limited to, site-specific mutagenesis

(Nucleic Acids Res. 10, 6487-6500, 1982). The number of mutated amino acids is not particularly limited as long as β-ionone ring-2-hydroxylase activity is retained. Usually, the number of mutated amino acids is within 30, preferably within 20, more preferably within 10, and most preferably within 5.

The peptide of (c) is a peptide which consists of an amino acid sequence having a 50% or more identity (ratio of identical amino acid sequence) with the peptide of (a) when the entire regions of both peptides are compared, and has β-ionone ring-2-hydroxylase activity. The "50% or more identity" is based on the following reason. Briefly, as described earlier in "Background Art", since the gene encoding an *Erwinia uredovora*-derived β-ionone ring-3-hydroxylase (CrtZ) was elucidated for the first time 14 years ago, structures of various CrtZ peptides have been elucidated. Among the CrtZ proteins which have been confirmed to have the same catalytic function, the enzyme that has the lowest homology to this *Erwinia uredovora*-derived CrtZ is a *Paracoccus zeaxanthinifaciens* (old designation: *Flavobacterium* sp. R1534)-derived CrtZ (Pasamontes, L., Hug, D., Tessier, M., Hohmann, H. P., Schierle, J., and van Loon, A. P., *Gene* 185, 35-41, 1997) with a 50% identity. Further, as described later, no CrtZ proteins have a 50% or more identity with the *Brevundimonas* sp. strain SD-212-derived peptide (CrtZ) consisting of a 161 amino acid sequence having β-ionone ring-3-hydroxylase activity as a result of search through DDBJ and GenBank databases. The CrtZ protein which has the highest identity with the *Brevundimonas* sp. strain SD-212-derived CrtZ is a CrtZ derived from *Erwinia herbicola* (recently, called *Pantoea agglomerans*) (JJBJ/GenBank accession no. M87280) with a 46% identity. Besides, according to the present invention, it was made clear that the catalytic functions of these two CrtZ proteins are identical. Taking into consideration that β-ionone ring-2-hydroxylase (CrtV) and β-ionone ring-3-hydroxylase (CrtZ) are enzymes very similar in nature, enzymes which consist of an amino acid sequence having a 50% or more identity with the peptide of (a) above and have β-ionone ring-2-hydroxylase activity (identical catalytic function) will surely be found in the future in microorganisms producing carotenoids in which position 2 of the β-ionone ring is hydroxylated. For example, such enzymes will be found by analyzing the genome of a bacterium producing carotenoids in which position 2 of the β-ionone ring is hydroxylated [e.g., *Erthrobacter* sp. strain PC6 (MBIC 02351)]. Further, the amino acid sequences of such enzymes which will be thus found in nature may be mutated by the

method described earlier.

The peptide of (d) is a bacterium-derived peptide which is obtainable by using DNA hybridization and has β-ionone ring-2-hydroxylase activity. The "stringent conditions" in the peptide of (c) means conditions which allow specific hybridization alone and eliminate non-specific hybridization. Usually, such conditions are about "1xSSC, 0.1% SDS, 37°C"; preferably about "0.5xSSC, 0.1% SDS, 42°C"; and more preferably about "0.2xSSC, 0.1% SDS, 65°C". The DNA obtainable by such hybridization usually has a high homology to the DNA represented by the nucleotide sequence as shown in SEQ ID NO: 3. The term "high homology" refers to a 60% or more homology, preferably a 75% or more homology, and still more preferably a 90% or more homology.

The gene of the present invention may be obtained, for example, as described below. First, a cosmid library of the marine bacterium *Brevundimonas* sp. strain SD-212 is prepared in *E. coli*. Then, the gene of the present invention may be obtained by such methods as colony hybridization using a homologous sequence of a carotenoid biosynthesis gene as described in Example 7 or PCR cloning.

Escherichia coli carrying plasmid p5Bre2-15 which was prepared by inserting into *E. coli* vector pBluescript II KS- a 12 kb *Eco*RI DNA fragment comprising the carotenoid biosynthesis gene cluster of *Brevundimonas* sp. strain SD-212 containing the gene of the invention, i.e., β-ionone ring-2-hydroxylase (*crtV*) gene, has been deposited at the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology under Accession No. P-19580.

4. Gene Encoding β-Ionone Ring-3-Hydroxylase (The Second Gene of the Invention)

The present invention includes the peptide of the following (e), (f) or (g).

(e) a peptide consisting of the amino acid sequence as shown in SEQ ID NO: 30;

(f) a peptide which consists of the amino acid sequence as shown in SEQ ID NO: 30 having addition, deletion or substitution of one or a plurality of amino acids and has β-ionone ring-3-hydroxylase activity; or

(g) a bacterium-derived peptide which is encoded by a DNA consisting of the nucleotide sequence as shown in SEQ ID NO: 29 or a DNA hybridizable to a complementary DNA to said DNA under stringent conditions and has β-ionone ring-3-hydroxylase activity.

The peptide of (e) is a *Brevundimonas* sp. strain SD-212-derived peptide (sometimes called "CrtZ") having β-ionone ring-3-hydroxylase activity and consisting of a

161 amino acid sequence.

The peptide of (f) is a peptide of (e) into which mutations that would not eliminate β-ionone ring-3-hydroxylase activity have been introduced. Such mutations include mutations occurring spontaneously in nature and artificial mutations. Means to cause artificial mutations is as described above.

The peptide of (g) is a bacterium-derived peptide which is obtainable by using DNA hybridization and has β-ionone ring-3-hydroxylase activity. The "stringent conditions" in the peptide of (g) are as described above.

5. Gene Encoding Geranylgeranyl Pyrophosphate Synthase (The Third Gene of the Invention)

The present invention also includes a gene encoding the following peptide (h), (i) or (j).

(h) a peptide consisting of the amino acid sequence as shown in SEQ ID NO: 32;

(i) a peptide which consists of the amino acid sequence as shown in SEQ ID NO: 32 having addition, deletion or substitution of one or a plurality of amino acids and has geranylgeranyl pyrophosphate synthase activity; or

(j) a bacterium-derived peptide which is encoded by a DNA consisting of the nucleotide sequence as shown in SEQ ID NO: 31 or a DNA hybridizable to a complementary DNA to the above DNA under stringent conditions and has geranylgeranyl pyrophosphate synthase activity.

The peptide of (h) is a *Brevundimonas* sp. strain SD-212-derived peptide (sometimes called "CrtE") having geranylgeranyl pyrophosphate synthase activity and consisting of a 298 amino acid sequence.

The peptide of (i) is a peptide of (h) into which mutations that would not eliminate geranylgeranyl pyrophosphate synthase activity have been introduced. Such mutations include mutations occurring spontaneously in nature and artificial mutations. Means to cause artificial mutations is as described above.

The peptide of (j) is a bacterium-derived peptide which is obtainable by using DNA hybridization and has geranylgeranyl pyrophosphate synthase activity. The "stringent conditions" in the peptide of (j) are as described above.

6. Microorganism Capable of Introducing a Hydroxyl Group at the Position 2(2') or 3(3') Carbon of β-Ionone Ring

The present invention includes a microorganism obtainable by introducing thereinto the β-ionone ring-2-hydroxylage gene described in 3 above, wherein the microorganism is capable of introducing a hydroxyl group at the position 2 carbon of β-ionone ring.

The present invention also includes a microorganism obtainable by introducing thereinto the β-ionone ring-3-hydroxylage gene described in 4 above, wherein the microorganism is capable of introducing a hydroxyl group at the position 3 carbon of β-ionone ring.

Not only the gene of the invention but also other carotenoid biosynthesis genes are often introduced into a microorganism. However, when the microorganism inherently has other carotenoid biosynthesis genes, other carotenoid biosynthesis genes may not be introduced or only a part of them is introduced.

Examples of host microorganisms include, but are not limited to, *E. coli*.

The "other carotenoid biosynthesis genes" include all or a part of a gene cluster required for synthesizing β-ionone ring-containing carotenoids from farnesyl pyrophosphate (FPP). Specific examples of such a gene cluster include, but are not limited to, *crtE* encoding an enzyme that synthesizes geranylgeranyl pyrophosphate (GGPP) from FPP, *crtB* encoding an enzyme that synthesizes phytoene from two molecules of GGPP, *crtI* encoding an enzyme that synthesizes lycopene from phytoene, *crtY* (usually, derived from *Erwinia* bacteria) encoding an enzyme that synthesizes β-carotene from lycopene, and *crtW* (usually, derived from *Paracoccus* bacteria) encoding β-ionone ring -4-ketolase.

When all or a part of such a gene cluster is integrated into an appropriate expression vector and then introduced into a host microorganism, the recombinant microorganism begins to produce β-ionone ring-containing carotenoids. (Every microorganism is capable of producing the substrate FPP. Although some microorganisms produce only a small amount of GGPP, every microorganism is also capable of producing GGPP.) When the first gene of the invention (*crtV* encoding β-ionone ring-2-hydroxylase) is further introduced and expressed in the above recombinant microorganism producing β-ionone ring-containing carotenoids, the microorganism begins to produce carotenoids in which a hydroxyl group is introduced at position 2(2'). When the second gene of the invention (*crtZ* encoding β-ionone ring-3-hydroxylase) is further

introduced and expressed in the above recombinant microorganism producing β-ionone ring-containing carotenoids, the microorganism begins to produce carotenoids in which a hydroxyl group is introduced at position 3(3').

Information about vectors of various microorganisms such as *E. coli* or yeast, and methods of introduction/expression of exogenous genes are disclosed in a number of experimental manuals (e.g., Sambrook, J., Russel, D. W., "Molecular Cloning: A Laboratory Manual, 3rd Edition", CSHL Press, 2001). Selection of vectors and introduction/expression of genes may be performed according to those manuals.

7. Method of Preparing 2(2')- or 3(3')-Hydroxylated Carotenoids

The present invention also includes a method of preparing a hydroxylated carotenoid, comprising culturing the above-described microorganism in a medium and obtaining from the resultant culture or cells a carotenoid in which the position 2 or 3 carbon of its β-ionone ring is hydroxylated.

Specific examples of carotenoids which are hydroxylated at the position 2 carbon of the β-ionone ring include, but are not limited to, β,β-carotene-2-ol (2-hydroxy-β-carotene), β,β-carotene-2,2'-diol (2,2'-dihydroxy-β-carotene), caloxanthin (2-hydroxyzeaxanthin), nostoxanthin (2,2'-dihydroxyzeaxanthin), 2-hydroxy-β,β-carotene-4,4'-dione (2-hydroxycanthaxanthin), 2,2'-dihydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxycanthaxanthin), 2-hydroxyastaxanthin and 2,3,2',3'-tetrahydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxyastaxanthin). Of the above carotenoids, 2-hydroxy-β,β-carotene-4,4'-dione (2-hydroxycanthaxanthin) and 2,2'-dihydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxycanthaxanthin) (especially the latter) have been demonstrated to have strong inhibitory effects on peroxidation of lipids as a result of *in vitro* experiments.

Specific examples of carotenoids which are hydroxylated at the position 3 carbon of the β-ionone ring include, but are not limited to, β-cryptoxanthin, zeaxanthin, caloxanthin (2-hydroxyzeaxanthin), nostoxanthin (2,2'-dihydroxyzeaxanthin), 2-hydroxyastaxanthin and 2,3,2',3'-tetrahydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxyastaxanthin).

8. Antioxidant

Since 2-hydroxy-β, β-carotene-4,4'-dione and 2,2'-dihydroxy-β,

β-carotene-4,4'-dione have strong inhibitory effects on peroxidation of lipids as described above, they may be used as an antioxidant for lipids, or even as an antioxidant for substances in general.

The antioxidant of the invention may be prepared by simply diluting the above-mentioned two carotenoids (active ingredients) with water. Preferably, the carotenoids are prepared into emulsions. Emulsions may be prepared by mixing and emulsifying aqueous components and oil-soluble components by conventional methods. Examples of aqueous components which may be used for this purpose include gallic acid, ascorbic acid, gums and vitamin P (flavonoids). Examples of oil-soluble components which may be used for this purpose include glycerin fatty acid ester and fats and oils such as rapeseed oil, soybean oil or corn oil. The contents of the above-mentioned two carotenoids in the antioxidant of the invention may be selected freely considering the product to which the antioxidant is to be added. Preferably, the content is 0.05-5%.

The antioxidant of the invention is useful mainly as an antioxidant component for foods, cosmetics and pharmaceuticals because of its safety and ability to inhibit oxidation. The doses of the above-mentioned two carotenoids as an antioxidant may vary depending on the product to which the antioxidant is to be added. For example, the carotenoid may be added at about 0.1 g per kg of foods and at about 0.05 g per kg of cosmetics.

Although the antioxidant of the invention produces sufficient effect when used alone, the antioxidant may be used in combination with conventional natural antioxidants (e.g., antioxidant whose active ingredient is tocopherol).

The SEQ ID NOS in the SEQUENCE LISTING of the present specification represent the following sequences.

SEQ ID NO: 1: a partial sequence of the *crtI* gene of *Brevundimonas* sp. strain SD-212.

SEQ ID NO: 2: the sequence of a 12 kb fragment cut out from pCos5-2 with *Eco*RI.

SEQ ID NO: 3: the sequence of ORF11 (presumed to be a β-ionone ring-2-hydroxylase gene) contained in the above-mentioned *Eco*RI fragment.

SEQ ID NO: 4: the amino acid sequence encoded by ORF11.

SEQ ID NO: 5: a primer (forward) for amplifying ORF1.

SEQ ID NO: 6: a primer (reverse) for amplifying ORF1.

SEQ ID NO: 7: a primer (forward) for amplifying *crtW*.

SEQ ID NO: 8: a primer (reverse) for amplifying *crtW*.

SEQ ID NO: 9: a primer (forward) for amplifying *crtY*.

SEQ ID NO: 10: a primer (reverse) for amplifying *crtY*.

SEQ ID NO: 11: a primer (forward) for amplifying *crtI*.

SEQ ID NO: 12: a primer (reverse) for amplifying *crtI*.

SEQ ID NO: 13: a primer (forward) for amplifying *crtB*.

SEQ ID NO: 14: a primer (reverse) for amplifying *crtB*.

SEQ ID NO: 15: a primer (forward) for amplifying ORF6.

SEQ ID NO: 16: a primer (reverse) for amplifying ORF6.

SEQ ID NO: 17: a primer (forward) for amplifying ORF7.

SEQ ID NO: 18: a primer (reverse) for amplifying ORF7.

SEQ ID NO: 19: a primer (forward) for amplifying *crtE*.

SEQ ID NO: 20: a primer (reverse) for amplifying *crtE*.

SEQ ID NO: 21: a primer (forward) for amplifying *idi*.

SEQ ID NO: 22: a primer (reverse) for amplifying *idi*.

SEQ ID NO: 23: a primer (forward) for amplifying *crtZ*.

SEQ ID NO: 24: a primer (reverse) for amplifying *crtZ*.

SEQ ID NO: 25: a primer (forward) for amplifying ORF11.

SEQ ID NO: 26: a primer (reverse) for amplifying ORF11.

SEQ ID NO: 27: a primer (forward) for amplifying ORF12.

SEQ ID NO: 28: a primer (reverse) for amplifying ORF12.

SEQ ID NO: 29: the sequence of *crtZ* (presumed to be a β-ionone ring-3-hydroxylase gene) contained in the above-mentioned *Eco*RI fragment.

SEQ ID NO: 30: the amino acid sequence encoded by *crtZ*.

SEQ ID NO: 31: the sequence of *crtE* (presumed to be a geranylgeranyl pyrophosphate synthase gene) contained in the above-mentioned *Eco*RI fragment.

SEQ ID NO: 32: the amino acid sequence encoded by *crtE*.

Effect of the Invention

Many of those carotenoids in which the 2(2') carbon of the β-ionone ring is

hydroxylated occur only in trace amounts in nature, and some have not yet been discovered. According to the present invention, it becomes possible to produce such carotenoids in large quantities. Further, the present invention also provides a gene capable of producing carotenoids in which the 3(3') carbon of the β-ionone ring is hydroxylated and a gene which synthesizes a carotenoid precursor geranylgeranyl pyrophosphate.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram showing the functions and synthesis pathway of existing carotenoid biosynthesis genes (enzymes).

Fig. 2 is a diagram showing the carotenoids produced by *Brevundimonas* sp. strain SD-212 and an estimated synthesis pathway for those carotenoids.

Fig. 3 shows the results of Southern hybridization (*Eco*RI digestion) using a *crtI* fragment as a probe [M: size marker (λ/*Hin*dIII-φX174/HaeIII digest); lanes 5-1 to 10-1: cosmid clones; SCS: cosmid vector SuperCos1; SD212: SD-212 chromosomal DNA]

Fig. 4 shows the results of Southern hybridization (*Bam*HI and *Bam*HI/*Eco*RI digestion) using a *crtI* fragment as a probe [M: size marker (λ/*Hin*dIII-φX174/HaeIII digest); lanes 5-2 to 9-1: cosmid clones; SD212: SD-212 chromosomal DNA; SCS: *Bam*HI/*Eco*RI digest of cosmid vector SuperCos1].

Fig. 5 shows the structure of the carotenoid biosynthesis gene cluster (12 kb EcoRI fragment) of *Brevundimonas* sp. strain SD-212.

Fig. 6 shows the results of HPLC-PDA analysis using pACCAR16ΔcrtX (plasmid for β-carotene production)-introduced *E. coli* as a host. a) HPLC chromatogram of pigments produced by pACCAR16ΔcrtX-introduced *E. coli* (470 mn). b) HPLC chromatogram of pigments produced by pUCBre-O11 and pACCAR16ΔcrtX-introduced *E. coli* (470 mn). The peaks of novel pigments are marked with arrows. c) HPLC chromatogram when zeaxanthin was added to b) above (470 nm).

Fig. 7 shows the results of HPLC-PDA analysis using pACCAR25ΔcrtX (plasmid for zeaxanthin production)-introduced *E. coli* as a host. a) HPLC chromatogram of pigments produced by pACCAR25ΔcrtX-introduced *E. coli* (470 mn). b) HPLC chromatogram of pigments produced by pUCBre-O11 and pACCAR25ΔcrtX-introduced *E. coli* (470 mn). The peaks of novel pigments are marked with arrows. c) HPLC chromatogram when zeaxanthin was added to b) above (470 nm). Peaks 1 and 2 were

identified as nostoxanthin and caloxanthin, respectively.

Fig. 8 shows the results of HPLC-PDA analysis using pAC-Cantha (plasmid for canthaxanthin production)-introduced *E. coli* as a host. a) HPLC chromatogram of pigments produced by pAC-Cantha-introduced *E. coli* (470 mn). b) HPLC chromatogram of pigments produced by pUCBre-O11 and pAC-Cantha-introduced *E. coli* (470 mn). The peaks of novel pigments are marked with arrows. c) HPLC chromatogram when astaxanthin was added to b) above (470 nm). Peaks 3 and 4 were identified as 2,2'-dihydroxy-β,β-carotene-4,4'-dione and 2-hydroxy-β,β-carotene-4,4'-dione, respectively.

Fig. 9 shows the results of HPLC-PDA analysis using pAC-Asta (plasmid for astaxanthin production)-introduced *E. coli* as a host. a) HPLC chromatogram of pigments produced by pAC-Asta-introduced *E. coli* (470 mn). b) HPLC chromatogram of pigments produced by pUCBre-O11 and pAC-Asta-introduced *E. coli* (470 mn). The peak of a novel pigment is marked with an arrow. Peak 2 was identified as 2,3,2',3'-tetrahydroxy-β,β-carotene-4-one. c) HPLC chromatogram when astaxanthin was added to b) above (470 nm). Peak 5 was identified as 2-hydroxyasataxanthin.

Fig. 10 is a diagram showing carotenoids produced by a recombinant *E. coli* and estimated synthesis pathways for them.

BEST MODE FOR CARRYING OUT THE INVENTION

Herein below, the present invention will be described in more detail with reference to the following Examples. However, the present invention is not limited to these Examples.

[EXAMPLE 1] Strains, Plasmids and Growth Conditions

The strains and plasmids used in the invention are shown in Table 1. The strains were cultured at 30°C in LB (Luria-Bertani) medium or 2xYT medium (Sambrook et al., 1989). When necessary, ampicillin (Ap, 100 μg/ml) or chloramphenicol (Cm, 20 μg/ml) was added to the medium.

A plasmid for canthaxanthin production (pAC-Cantha) and a plasmid for astaxanthin and adonixanthin (4-ketozeaxanthin) production (pAC-Asta) were constructed as described below.

Briefly, *Paracoccus* sp. MBIC 01143 (*Agrobacterium aurantiacum*)-derived *crtW*

gene was synthesized so that the codon usage is consistent with in *GAP* gene in yeast *Candida utilis.* The amino acid sequence encoded by the resultant *crtW* was made identical with the amino acid sequence of the original CrtW. This method is described previously (Miura et al., 1998). With this totally synthesized *crtW* sequence as a template, PCR was performed using H1437 primer [containing *Ava*I site (underlined) and SD sequence (positions 10-15 of H1437)] and H1438 primer [containing *Not*I site (underlined)]. The resultant PCR product was digested with *Ava*I and *Not*I to obtain a 0.76 kb *Ava*I-*crt*W-*Not*I fragment.

H1437: 5'-GTCCCGAGAAGGAGGCTAGATATGTCCGCTCACGCTTTGC-3'

H1438: 5'-CGGCGGCCGCCCGGGACTAAGCGGTGTCACCCTTGGTTCT-3'

With plasmid pCAR16 (Misawa et al., 1990) as a template, PCR was performed using H1431 primer [containing *Not*I site (underlined) and SD sequence (positions 16-21 of H1431)] and H1432 primer [containing *Sal*I site (underlined)]. The resultant PCR product was digested with *Not*I and *Sal*I to obtain a 1.1 kb *Not*I-*crtE*-*Sal*I fragment.

H1431: 5'-ATGCGGCCGCTTATAAGGACAGCCCGAATG-3'

H1432: 5'-CAGTCGACATCCTTAACTGACGGCAGCGAG-3'

The above-described 0.76 kb *Ava*I-*crt*W-*Not*I fragment and 1.1 kb *Not*I-*crtE*-*Sal*I fragment were ligated to each other via the *Not*I sites and then ligated to a large fragment containing *crtY*, *crtI* and *crtB* which had been obtained by *Ava*I/*Sal*I digestion of pACCAR16ΔcrtX, to thereby obtain plasmid pAC-Cantha.

Subsequently, the above-described 0.76 kb *Ava*I-*crt*W-*Not*I fragment and 1.1 kb *Not*I-*crtE*-*Sal*I fragment were ligated to each other via the *Not*I sites in the same manner as described above, and then ligated to a large fragment containing *crtY*, *crtI*, *crtB* and crtZ which had been obtained by *Ava*I/*Sal*I digestion of pACCAR25ΔcrtX, to thereby obtain plasmid pAC-Asta.

Table 1

Strains and Plasmids used in the Present Invention

Strain/Plasmid	Nature*	Reference/Manufacturer
Strain		
Brevundimonas sp. MBIC03018	2-Hydroxylated carotenoid-producing bacterium (strain SD-212)	Yokoyama et al, 1996
Escherichia coli XL1-Blue MR	Host of cosmid vector SuperCos 1	Stratagene
E. coli DH5 α	Host for genetic engineering experiments	TOYOBO
Plasmid		
pACCAR16 Δ crtX	Cm^r; plasmid comprising *crtE*, *crtB*, *crtI* and *crtY*	Misawa et al, 1995
pACCAR25 Δ crtX	Cm^r; plasmid comprising *crtE*, *crtB*, *crtI*, *crtY* and *crtZ*	Misawa et al, 1995
pAC-Cantha	Cm^r; plasmid comprising *crtE*, *crtB*, *crtI*, *crtY* and *crtW*	Present invention
pAC-Asta	Cm^r; plasmid comprising *crtE*, *crtB*, *crtI*, *crtY*, *crtZ* and *crtW*	Present invention
SuperCos 1	Ap^r; cosmid vector	Stratagene
pBluescript II KS-	Ap^r; cloning vector	TOYOBO
pGEM-T Easy	Ap^r; cloning vector	Promega
pUC18	Ap^r; cloning vector	TOYOBO
pCos5-2	Ap^r; *Brevundimonas* sp. MBIC03018-derived 47 kb DNA fragment (partially digested with *Sau*3AI) is inserted at the *Bam*HI site of SuperCos 1	Present invention
CRTI-SD212	Ap^r; *Brevundimonas* sp. MBIC03018-derived *crtI* is PCR amplified and inserted into pGEM-T Easy	Present invention
p5Bre2-15	Ap^r; pCos5-2-derived 12 kb *Eco*RI fragment is inserted into pBluescript II KS-	Present invention
pUCBre-O11	Ap^r; p5Bre2-15-derived 2-hydroxylase gene is PCR amplified and inserted into pUC18	Present invention

Ap^r: ampicillin resistance; Cm^r: chloramphenicol resistance

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Sambrook, J., Fritsch, E. F., and Maniatis T. 1989. "Molecular cloning: a laboratory manual. 2nd ed.", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

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Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Saito, T., Ohtani, T., and Miki, W., "Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level", J. Bacteriol. 177, 6575-6585, 1995 (Non-Patent Document 1)

[EXAMPLE 2] Genetic Engineering Experiments

Conventional genetic engineering experiments such as construction of plasmids, treatment with restriction enzymes, ligation reaction and transformation were conducted according to the methods disclosed in Sambrook et al. (1989), *op. cit.*

[EXAMPLE 3] Preparation of Chromosomal DNA from *Brevundimonas* sp. Strain SD-212

Brevundimonas sp. strain SD-212 (SD212; MBIC 03018) was cultured in 300 ml of Marine Broth (MB) medium (Difco) at 25°C for 3 days. Cells were harvested, washed with STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) twice, thermally treated at 68°C for 15 min and then suspended in solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) containing 5 mg/ml lysozyme (Sigma) and μg/ml RNase A (Sigma). After 1 hr incubation at 37°C, Protenase K (Sigma) was added thereto to give a concentration of 250 μg/ml, followed by incubation at 37°C for 10 min. N-Lauroylsarcosin-Na was added thereto to give a final concentration of 1% and mixed gently and thoroughly by inverting, followed by incubation at 37°C for 3 hr. After several times of phenol/chloroform extraction, while adding two volumes of ethanol slowly, chromosomal DNA deposited was wound around a glass rod and rinsed with 70% ethanol. Then, the DNA was dissolved in 2 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to prepare a chromosomal DNA solution.

[EXAMPLE 4] Amplification of Partial Fragment of Phytoene Desaturase Gene (*crtI*) by PCR

A partial fragment of phytoene desaturase gene (*crtI*) was amplified by PCR using crtI-Fo primer (5'-TTY GAY GCI GGI CCI ACI GT -3') and crtI-Re primer (5'-CCI GGR TGI GTI CCI GCI CC-3') (which had been designed utilizing the homology of *crtI* genes among carotenoid producing bacteria) and the chromosomal DNA from *Brevundimonas* sp. strain SD-212 obtained as described above as a template. As a thermal resistance DNA polymerase, La-Taq (TaKaRa) was used. After thermal denaturation at 96°C for 5 min, 35 cycles of 98°C for 20 sec, 58°C for 30 sec and 72°C for 1 min were carried out. The

amplified products were confirmed by 1% agarose gel electrophoresis. Then, a 1.1 kb DNA fragment was cut out from the gel and purified with Qiagen Gel Extraction kit (QIAGEN) or Gene Clean II Kit (BIO101). The purified DNA fragment was ligated to pGEM-T Easy and transformed into *E. coli* (DH5α). This plasmid was designated pCRTI-SD212. The *E. coli* was cultured in 2 ml of ampicillin-added LB liquid medium at 37°C overnight, followed by extraction of the plasmid. The nucleotide sequence (partial) of the extracted plasmid was determined using a DNA sequencing kit (Big Dye Terminator Cycle Sequencing Ready Reaction Kit ver.2; Perkin-Elmer) and a model 3700 DNA sequencer (Perkin-Elmer) according to the protocol attached. The thus determined DNA sequence (SEQ ID NO: 1) was subjected to homology search using Blast (Altschul and Lipman, 1990). As a result, it was confirmed that this DNA sequence has a homology to phytoene desaturase gene (*crtI*). A part of the PCR amplified and purified DNA fragment was used as a probe in the colony hybridization and Southern hybridization conducted in Examples 7 and 8.

Altschul, S. F. and Lipman, D. J., "Protein database search for multiple alignments", Proc. Natl. Acad. Sci. USA 87, 5509-5513, 1990.

[EXAMPLE 5] Construction of Cosmid Library

Experimental procedures up to acquisition of phage particles from a prepared solution of *Brevundimonas* sp. strain SD-212 chromosomal DNA were according to the instructions attached to SuperCos 1 Cosmid Vector Kit (Stratagene). Briefly, the chromosomal DNA from *Brevundimonas* sp. strain SD-212 was partially digested with *Sau*3AI and ligated to the *Bam*HI site of the cosmid vector, followed by packaging into phage particles using LAMBDA INN (Nippon Gene). Subsequently, *E. coli* XL1-Blue MR and pACCAR25ΔcrtX-carrying *E. coli* XL1-Blue MR that produces zeaxanthin were infected with the resultant phage particles. On Ap containing LB plates and Ap+Cm containing LB plates, approx. 1000 Ap resistant colonies and approx. 1000 Ap+Cm resistant colonies were obtained, respectively. The resultant colonies were transferred onto fresh antibiotic containing LB plates with sterilized toothpicks. At this stage, no colonies were obtained which exhibit a color change.

This cosmid vector SuperCos 1 (Stratagene) is a 7.9 kb vector and capable of insertion of a 30-45 kb DNA fragment. This cosmid vector has the following advantages. Since it has two cos regions, efficient packaging is possible. Dephosphorylation

operation for preventing the packaging of cosmid concatemers is unnecessary. Since it is possible to dephosphorylate the chromosomal DNA to be inserted, mixing of re-ligated fragments of the chromosomal DNA fragment need not be worried about. Size fractionation is also unnecessary.

[EXAMPLE 6] Attempt of Expression Cloning

Using 700 colonies from the cosmid library constructed in Example 5 using the pACCAR25ΔcrtX-carrying and zeaxanthin-producing *E. coli* as a host, a 2 ml aliquot of each colony was cultured. Carotenoid pigments were extracted therefrom with acetone, followed by analysis of the carotenoids with HPLC-PDA (photodiode array detector). The procedures are described in Example 11. Whether novel carotenoid(s) was/were produced in addition to the control zeaxanthin was examined. As a result, no colonies were obtained that synthesize novel carotenoid(s). Therefore, the inventors concluded that expression cloning of the carotenoid biosynthesis genes of *Brevundimonas* sp. strain SD-212 is impossible.

[EXAMPLE 7] Colony Hybridization

Using 500 colonies from the cosmid library constructed in Example 5 using *E. coli* XL1-Blue MR as a host and the partial fragment of phytoene desaturase gene (*crtI*) amplified in Example 4 by PCR as a probe, colony hybridization was performed to thereby screen for *crtI*-containing clones. First, the *E. coli* was seeded on plates and cultured at 37°C. At this time, 48 colonies were seeded per plate. After overnight culture, a Hybond-N+ membrane 82 mm in diameter (Amersham Pharmacia) was placed on the plate and a mark was put on the membrane an injection needle. The membrane was peeled off and placed with the surface which cells adhered onto upward. The membrane was incubated for 5 min with a 3 mm filter paper (Whatman) containing 10% SDS solution and incubated for another 5 min with a 3 mm filter paper containing a denaturing solution (1.5 M NaCl, 0.5 M NaOH). Then, the membrane was dipped in a neutralizing solution (1.5 M NaCl, 0.5M Tris-HCl) for 5 min (twice). Further, the membrane was washed with 2xSSC twice. At this time, the membrane was wiped off strongly with Kimtowel so that no cell debris remained on the membrane. After these treatments, the membrane was air-dried on Kimtowel and Kimwipe for 30 min and baked at 80°C for 2 hr to thereby immobilize DNA on the membrane. A DNA probe was prepared using Alkphos Direct Labeling and Detection System (Amersham Pharmacia) according to the manufacturer's

protocol and used in colony hybridization. As a result of this colony hybridization using the partial fragment of phytoene desaturase gene (*crtI*) as a probe DNA, 6 positive clones were obtained from the 500 colonies used. The plasmids contained in these 6 clones were designated pCos5-1, pCos5-2, pCos7-1, pCos8-1, pCos9-1 and pCos10-1, respectively.

[EXAMPLE 8] Southern Hybridization

The 6 positive clones selected in Example 7 were cultured in 2 ml of Ap-added LB liquid medium at 37°C overnight, followed by extraction of plasmid DNA. The extracted plasmid DNA was completely digested with *Eco*RI by incubating at 37°C for several hours and then electrophoresed. As controls, vector SuperCos 1 and similarly digested *Brevundimonas* sp. strain SD-212 chromosomal DNA were used. Electrophoresis was performed in a small submarine-type electrophoresis bath Mupid (Cosmobio) using 1% agarose gel at 50 V for about 70 min. As an electrophoresis buffer, 1xTBE buffer was used. After the electrophoresis, the gel was stained with ethidium bromide and decolored with ultra-pure water. Then, photographs were taken under UV radiation (Fig. 3). Subsequently, the DNA was transferred onto a nylon membrane (Hybond N+) by capillary blotting with 0.4M NaOH solution. After this treatment, the membrane was baked at 80°C for 2 hr to thereby immobilize the DNA on the membrane. Subsequently, Southern hybridization was performed using Alkphos Direct Labeling and Detection System (Amersham Pharmacia) according to the protocol attached to the kit. As a probe DNA, the above-described partial fragment of phytoene desaturase gene (*crtI*) was used. As a result, 3 clones (pCos5-2, pCos7-1 and pCos9-1) out of the 6 positive clones showed a 12 kb *Eco*RI fragment-positive signal (Fig. 3). The control SD-212 chromosomal DNA exhibited a smear band on the high molecular side as a result of the electrophoresis; this DNA was little digested. Although slightly, a positive signal was recognized on the high molecular side. One of the reasons for this little digestion may be that the chromosomal DNA had been partially methylated and this inhibited the degradation by *Eco*RI. Further, a similar experiment was conducted in which the plasmids of the 3 positive clones, SuperCos 1, and the chromosomal DNA from *Brevundimonas* sp. strain SD-212 were digested with *Bam*HI or *Bam*HI-*Eco*RI (Fig. 4). The results revealed that a 9 kb DNA fragment-positive signal was recognized when digested with *Bam*HI, and a 8.2 kb DNA fragment-positive signal was recognized when digested with *Bam*HI-*Eco*RI. Although weak, positive signals were also recognized at the same positions in the chromosomal

DNA from *Brevundimonas* sp. strain SD-212.

[EXAMPLE 9] Analysis of a Carotenoid Gene Cluster

Of the 3 positive clones selected in Example 8 (pCos5-2, pCos7-1 and pCos9-1), pCos5-2 was used in this experiment. The 12 kb insert was cut out with *Eco*RI, ligated to the *Eco*RI site of plasmid vector pBluescript II KS-, and transformed into *E. coli* DH5α. This plasmid was designated p5Bre2-15. The resultant *E. coli* was cultured in 2 ml of Ap-added LB liquid medium at 37°C overnight, followed by extraction of plasmid. The nucleotide sequence of the extracted plasmid was sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kit ver.2 (Perkin-Elmer) and model 3700 DNA sequencer (Perkin-Elmer) according to the manufacturer's protocol. Gene-coding regions of the thus determined DNA sequence (SEQ ID NO: 2) were estimated using GeneMark.hmm (Lukashin A. and Borodovsky M.) and SD-like sequences were confirmed. As a result, 12 open reading frames (ORFs) were found in the 12 kb fragment (Fig. 5). Individual ORFs were subjected to homology search at the amino acid level using Blast. The results revealed that 7 ORFs out of 12 show homology to existing carotenoid biosynthesis genes (*crtW, crtY, crtI, crtB, crtE, crtZ* and *idi*) (Table 2). The remaining 5 genes (ORFs) were unknown genes having no overall homology to any existing gene. The search of identity (the ratio of identical amino acid sequence) between the *Brevundimonas* sp. strain SD-212-derived 7 Crt enzymes (including one Idi) showing homology to known carotenoid biosynthesis enzymes and other organism-derived corresponding enzymes was performed as described below to ensure accuracy. Briefly, using Blast program (ver. 2) (Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipmann, D. J., *Nucleic Acids Res.* 25, 3389-3402, 1997), homology search was carried out through GenBank/DDBJ databases. Subsequently, using the hit amino acid sequences, individual Crt proteins were analyzed with Clustal W program (ver. 1.8) (Higgins, D. G., Thompson, J. D., and Gibson, T. J., *Methods Enzymol.* 266, 383-402, 1996) and GeneDoc program (Nicholas, K. B., Nicholas H. B. Jr., and Deerfield, D. W. II., *Embnew. News* 4, 14, 1997) to thereby determine the identity between entire regions. Table 2 shows the identities between individual *Brevundimonas* sp. strain SD-212-derived 7 Crt enzymes (including one Idi) and other bacterium-derived Crt proteins that showed the highest homology thereto. The results of this analysis revealed that the *Brevundimonas* sp. strain SD-212-derived 7 Crt enzymes (including one Idi) show

only moderate or low homology to known, other organism-derived corresponding proteins. As seen from Table 2, even in the SD-212-derived CrtI which showed the highest homology, the identity was 72%. More surprisingly, the SD-212-derived CrtZ and CrtE have no known, other organism-derived corresponding proteins showing more than 50% identity. Even at the highest, the identities were 46% and 39%, respectively. The *crtZ* gene of SD-212 is encoded by the nucleotides from positions 1319 to 1804 of the complementary strand of the 12 kb *Eco*RI fragment (11,991 kb) as shown in SEQ ID NO: 2. The nucleotide sequence of this *crtZ* gene is shown in SEQ ID NO: 29, and the amino acid sequence encoded thereby (CrtZ) is shown in SEQ ID NO: 30. The *crtE* gene of SD-212 is encoded by the nucleotides from positions 2963 to 3859 of the complementary strand of the 12 kb *Eco*RI fragment (11,991 kb) as shown in SEQ ID NO: 2. The nucleotide sequence of this *crtE* gene is shown in SEQ ID NO: 31, and the amino acid sequence encoded thereby (CrtE) is shown in SEQ ID NO: 32. Further, examination of the locations of individual *crt* genes (i.e., locations of *crtW* and *crtZ*, orientations of other genes, etc.) revealed that the carotenoid biosynthesis gene cluster from SD-212 is greatly different in structure from the previously reported carotenoid biosynthesis gene clusters of bacteria producing carotenoids with hydroxylated β-ionone ring(s) (Misawa et al., 1990 & 1995; Hannibal et al., 2000) (Fig. 5). An IPP isomerism gene (*idi*) present within a carotenoid biosynthesis gene cluster was found for the first time. By the way, *E. coli* carrying plasmid p5Bre2-15 containing all of the above-described 12 ORFs (including 7 *crt* genes) was unable to produce any carotenoid. Therefore, it has become clear that the carotenoid biosynthesis gene cluster of *Brevundimonas* sp. strain SD-212 is not capable of functional expression as it is.

Table 2

Characteristics and Predicted Functions of the Various ORFs Present in the Carotenoid Biosynthesis Gene Cluster in *Brevundimonas* sp. Strain SD-212

Designation of ORF	GC%	No. of Amino Acid Residues	Predicted Function	Homology to Gene Product of Other Organism (%)		GenBank number
ORF1	69.7	140	Unknown			
crtW	69.6	244	β -carotene C4 oxygenase	CrtW: *Brevundimonas aurantiaca*	(96%)	AAN86030
crtY	70.2	392	lycopene cyclase	CrtY: *Xanthobacter autotrophicus* Py2	(52%)	AF408848
crtI	67.3	493	phytoene desaturase	Crt I: *Xanthobacter autotrophicus* Py2	(72%)	AF408848
crtB	72	310	phytoene synthase	CrtB: *Xanthobacter autotrophicus* Py2	(53%)	AF408848
ORF6	75.8	354	Unknown			
ORF7	74.6	315	Unknown			
crtE	71	298	GGPP synthase	CrtE: *Xanthobacter autotrophicus* Py2	(39%)	AF408847
idi	74.9	350	Type II IPP isomerase	IPP isomerase: *Pantoea agglomerans*	(53%)	M87280
crtZ	66.9	161	β -carotene C3 hydroxylase	CrtZ: *Pantoea agglomerans*	(46%)	M87280
ORF11	70.7	257	Unknown			
ORF12	66.7	122	Unknown			

CrtW, *Brevundimonas aurantiaca* (GenBank number AAN86030); CrtY, CrtI, CrtB, CrtE, *Xanthobacter* sp. Py2 (GenBank no. AF408848, AF408847); IPP isomerase(Idi), CrtZ , *Pantoea agglomerans* (*Erwinia herbicola* Eho10) (GenBank no.M87280)

Lukashin A. and Borodovsky M., 1998, "GeneMark.hmm: new solutions for gene finding", NAR, Vol. 26, No. 4, pp. 1107-1115.

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Hannibal, L., Lorquin, J., D'Ortoli, N.A., Garcia, N., Chaintreuil, C., Masson-Boivin, C., Dreyfus, B. and Giraud, E., "Isolation and characterization of canthaxanthin biosynthesis genes from the photosynthetic bacterium *Bradyrhizobium* sp. strain ORS278", J. Bacteriol. 182, 3850-3853, 2000

Larsen, R.A., Wilson, M.M., Guss, A.M. and Metcalf, W.W., "Genetic analysis of pigment biosynthesis in Xanthobacter autotrophicus Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria", Arch. Microbiol. 178, 193-201, 2002

[EXAMPLE 10] Construction of β-Galactosidase Fusion Protein Expression Plasmids

In order to elucidate the functions of the ORFs, each ROF was amplified by PCR using the DNA of plasmid p5Bre2-15 as a template so that each ORF is expressed as a fusion protein with the lead sequence of β-galactosidase gene (*lacZ*) encoded by an *E. coli* vector pUC18 (TOYOBO). Thus, individual plasmids for expressing β-galactosidase fusion proteins were constructed. The inventors expected that each ORF would be functionally expressed in *E. coli* by this method. Specifically, individual ORFs were amplified with primers (as shown in SEQ ID NOS: 5-28) designed so that amplified products with 5' terminal *Eco*RI site and 3' terminal *Bam*HI or *Xba*I site could be obtained. As a thermal resistance DNA polymerase, La-Taq (TaKaRa) was used. After thermal denaturation at 96°C for 5 min, 35 cycles of 98°C for 20 sec, 56°C for 30 sec and 72°C for 1 min were carried out. A part of the amplified products was confirmed by 1% agarose gel electrophoresis. The remaining amplified product was ethanol-precipitated, digested with *Eco*RI + *Bam*HI or *Eco*RI + *Xba*I, and then confirmed by 1% agarose gel electrophoresis. Subsequently, the DNA fragment with an expected length was cut out from the gel and purified with Qiagen Gel Extraction kit (QIAGEN) or Gene Clean II Kit (BIO101). The purified DNA was ligated to the *Eco*RI-*Bam*HI or *Eco*RI-*Xba*I site of pUC18, and transformed into E. coli DH5α. These β-galactosidase fusion protein expression plasmids are designed so that a leader sequence of β-galactosidase consisting of 7 amino acids (Met Thr Met Ile Thr Asn Ser) is added before the inherent starting amino acid Met of each ORF.

[EXAMPLE 11] Expression of β-galactosidase-Individual Crt Fusion Protein Genes and Analysis of Pigment Production

E. coli clones carrying plasmids containing *crtE, crtB, crtI, crtY, crtZ* and *crtW* genes, respectively, out of the plasmids described in Example 10 were individually cultured in 2 ml of Ap-added LB liquid medium at 37°C overnight, followed by extraction of plasmid. The nucleotide sequence of the extracted plasmid was confirmed using Big Dye Terminator Cycle Sequencing Ready Reaction Kit ver.2 (Perkin-Elmer) and model 3700 DNA sequencer (Perkin-Elmer) according to the manufacturer's protocol. The individual plasmids were designated pUCBre-E (*lacZ::crtE*), pUCBre-B (*lacZ::crtB*),

pUCBre-I (*lacZ::crtI*), pUCBre-Y (*lacZ::crtY*), pUCBre-Z (*lacZ::crtZ*) and pUCBre-W (*lacZ::crtW*). Subsequently, these plasmids were introduced into *E. coli* clones carrying the various carotenoid producing plasmids (chloramphenicol (Cm) resistant) indicated at the left side in Table 3. Each of the resultant *E. coli* transformants was cultured in 2 ml of Ap and Cm-added LB liquid medium under induction by addition of 1 mM IPTG at 30°C for 48 hr. Then, cells were harvested by centrifugation and washed with STE twice. Acetone (200 μl) was added thereto and vortexed to thereby transfer pigments from cells to acetone. The resultant mixture was centrifuged. The supernatant was filtered and subjected to pigment analysis with an HPLC-PDA system (Waters Alliance 2695 and 2996 photodiode array detector). Using a TSK gel ODS-80Ts column (Tosoh), solvents were fed as described below. Solvent A (95% methanol) and solvent B [methanol: tetrahydrofuran (THF)=7:3]) were used. First, solvent A 100% was fed for 5 min, followed by a linear gradient from solvent A to solvent B for 5 min, and finally solvent B was fed for 8 min. Pigments were detected with a photodiode array detector and analyzed with an accessory software Empower. As standards, pigments extracted from the various carotenoid synthesizing *E. coli* clones (left side, Table 3) or synthesized products were used. By comparing retention times at 470 nm and absorption waveforms, it was confirmed various carotenoids were produced exactly as predicted (right side, Table 3). These results revealed clearly that Brevundimonas sp. strain SD-212-derived various fusion *crt* genes work in *E. coli* and have similar functions to those of the existing *crt* genes (*crtE, crtB, crtI, crtY, crtZ* and *crtW*). However, the expression of pUCBre-I (*lacZ::crtI*) と pUCBre-Y (*lacZ::crtY*) was considerably weak in *E. coli*. With respect to SD-212-derived CrtE and CrtZ which show only a 50% or less identity with known, other organism-derived Crt protein, it was confirmed by this experiment that they have strong catalytic activities in *E. coli*. It has become clear that this CrtE has the same catalytic activity as that of *Erwinia uredovora* (*Pantoea ananatis*)-derived CrtE. That is, this CrtE is a GGPP synthase generating geranylgeranyl pyrophosphate (GGPP) from farnesyl pyrophosphate (FPP). It has also become clear that the CrtZ is a β-ionone ring-3-hydroxylase (β-C3-hydroxylase; 3,3'-β-hydroxylase) which has an activity to generate zeaxanthin from β-carotene and to generate astaxanthin from canthaxanthin. Fig. 2 shows the carotenoids produced by *Brevundimonas* sp. strain SD-212. It is evident that SD-212-derived CrtZ is involved in the synthesis of all the carotenoids shown in Fig. 2 in

which position 3 of the β-ionone ring is hydroxylated (e.g., 2-hydroxyastaxanthin and 2-hydroxyadonixanthin).

Table 3

Identification of the Functions of Various *crt* Genes from *Brevundimonas* sp. Strain SD-212

Property of the recombinant *E. coli* used as a host		Identification of carotenoid pigments produced by double-recombinant *E. coli*	
Plasmid	Carotenoid to be accumulated	Plasmid SD-212-derived *crt* gene (*lacZ*::each *crt*)	Produced carotenoid
pACCAR25ΔcrtE(*crtB,I,Y,Z,X*)	FPP	pUCBre-E (*lacZ* ::*crtE*)	Zeaxanthin, its glucosides
pACCAR25ΔcrtB(*crtE,I,Y,Z,X*)	GGPP	pUCBre-B (*lacZ* ::*crtB*)	Zeaxanthin, its glucosides
pACCRT-EB(*crtE,B*)	Phytoene	pUCBre-I (*lacZ* ::*crtI*)	Lycopene (in trace amount)
pACCRT-EIB(*crtE,B,I*)	Lycopene	pUCBre-Y (*lacZ* ::*crtY*)	γ-Carotene (in trace amount)
pACCAR16ΔcrtX(*crtE,B,I,Y*)	β-carotene	pUCBre-Z (*lacZ*:: *crtZ*)	Zeaxanthin(80%), β-Cryptoxanthin(10%)
pAC-Cantha(*crtE,B,I,Y,W*)	Canthaxanthin	pUCBre-Z (*lacZ* ::*crtZ*)	Astaxanthin(41%), Adonixanthin(47%)
pACCAR16ΔcrtX(*crtE,B,I,Y*)	β-carotene	pUCBre-W (*lacZ* ::*crtW*)	Canthaxanthin (90%), Echinenone(5%)

[EXAMPLE 12] Expression of β-Galactosidase-ORF11 Fusion Protein and Analysis of Pigment Production

E. coli clones carrying plasmids containing ORF1, ORF6, ORF7, ORF11 and ORF12, respectively, out of the plasmids described in Example 10 were individually cultured in 2 ml of Ap-added LB liquid medium at 37°C overnight, followed by extraction of plasmid. The nucleotide sequence of the extracted plasmid was confirmed using Big Dye Terminator Cycle Sequencing Ready Reaction Kit ver.2 (Perkin-Elmer) and model 3700 DNA sequencer (Perkin-Elmer) according to the manufacturer's protocol. The individual plasmids were designated pUCBre-O1 (*lacZ*::SD212-ORF1), pUCBre-O6 (*lacZ*::SD212-ORF6), pUCBre-O7 (*lacZ*::SD212-ORF7), pUCBre-O11 (*lacZ*::SD212-ORF11) and pUCBre-O12 (*lacZ*::SD212-ORF12). Subsequently, these plasmids were introduced into *E. coli* clones carrying the various carotenoid producing plasmids (Cm resistant) indicated at the left side in Table 3. Each of the resultant *E. coli* transformants was cultured in 2 ml of Ap and Cm-added LB liquid medium under

induction by addition of 1 mM IPTG at 30°C for 48 hr. Then, cells were harvested by centrifugation and washed with STE twice. Acetone (200 μl) was added thereto and vortexed to thereby transfer pigments from cells to acetone. The resultant mixture was centrifuged. The supernatant was filtered and subjected to pigment analysis with an HPLC-PDA system in the same manner as described in Example 11.

As a result of the above experiment, plasmid pUCBre-O11-introduced *E. coli* alone produced a positive result (the nucleotide sequence of ORF11 is shown in SEQ ID NO: 3, and the amino acid sequence encoded thereby is shown in SEQ ID NO: 4). Briefly, in the clone which was obtained by introducing the ORF11 fusion protein expression plasmid pUCBre-O11 (*lacZ*::SD212-ORF11) into pACCAR16ΔcrtX-carrying, β-carotene-producing *E. coli* (DH5α), the resultant pigment extract exhibited the presence of β-carotene (451 nm, 478 nm) at retention time 16 min, the presence of a substance with absorption maximums (451.0 nm, 478.8 nm) at retention time 11 min on the higher polar side, and the presence of a substance with absorption maximums (452.2 nm, 477.6 nm) at retention time 13 min (Fig. 6; marked with arrows). However, the amounts of these conversion products were small. Since these substances may be zeaxanthin, co-HPLC was conducted by mixing the pigment extract solution with an acetone extract whose major component is pACCRT25ΔcrtX-derived zeaxanthin. The results revealed that the two peaks of these conversion products do not overlap with the peak of zeaxanthin (retention time: 10.6 min) (Fig. 6c) and thus they are substances different from zeaxanthin. The two carotenoids at retention times of 11 min and 13 min were identified as β,β-carotene-2,2'-diol (β,β-carotene-2,2'-diol; 2,2'-dihydroxy-β-carotene) and β,β-carotene-2-ol (β,β-caroten-2-ol; 2-hydroxy-β-carotene), respectively (see Fig. 10).

In the clone which was obtained by introducing the ORF11 fusion protein expression plasmid pUCBre-O11 (*lacZ*::SD212-ORF11) into pACCAR25ΔcrtX-carrying, zeaxanthin-producing *E. coli* (DH5α), the resultant pigment extract exhibited the presence of zeaxanthin (451 nm, 480 nm) at retention time 10.6 min. Other new peaks were also observed; substance 1 with absorption maximum (451.0 nm, 478.8 nm) at retention time 9.1 min and substance 2 with absorption maximums (452.2 nm, 477.6 nm) at retention time 9.9 min (Fig. 7; marked with arrows). Carotenoids 1 and 2 were identified as nostoxanthin (2,2'-dihydroxyzeaxanthin) and caloxanthin (2-hydroxyzeaxanthin), respectively (see Fig. 10 and Example 13). The above results demonstrate that the gene

product encoded by ORF11 is a β-ionone ring-2-hydroxylase (β-C2-hydroxylase; 2,2'-β-hydroxylase) which is an enzyme capable of introducing a hydroxyl group at the position 2 carbon of the β-ionone ring in carotenoids such as β-carotene and zeaxanthin.

Subsequently, plasmid pUCBre-011 was introduced into canthaxanthin-producing *E. coli* (DH5α) which carries plasmid pAC-Cantha containing the gene of an enzyme introducing a keto group at position 4 of β-ionone ring, and the resultant pigments were analyzed in the same manner as described in Example 11. As a result, a peak of canthaxanthin was recognized at retention time 10.7 min. Besides, there were recognized on the higher polar side a peak of substance 3 at retention time 4.7 min and a peak of substance 4 at retention time 8.7 min (Fig. 8; marked with arrows). The maximum absorption wavelengths were 478 nm and 474 nm, respectively. These waveforms suggested the presence of substances showing a single-mountain form typical in those carotenoids having keto group(s) in the conjugated system of the β-ionone ring. Further, the results of co-HPLC with astaxanthin (retention time: approx. 6.6 min) confirmed that these peaks are not astaxanthin (Fig. 8c). Carotenoids 3 and 4 were identified as 2,2'-dihydroxy-β,β-carotene-4,4'-dione (2,2'-dihyrroxycanthaxanthin) and 2-hydroxy-β,β-carotene-4,4'-dione (2-hydroxcanthaxanthin) (see Fig. 10 and Example 13).

Finally, plasmid pUCBre-O11 (*lacZ*::SD212-ORF11) was introduced into an *E. coli* clone carrying plasmid pAC-Asta and producing astaxanthin and adonixanthin, and the resultant pigments were analyzed in the same manner as described in Example 11 (Fig. 9). As a result, a peak of astaxanthin was observed at retention time 6.4-6.7 min and a peak of adonixanthin at retention time 8.4-8.6 min. Further, the presence of substance 5 with absorption maximum of 475 nm was observed on more highly polar side than astaxanthin whose retention time is 5.1-5.2 min.; this substance showed a waveform of single-mountain form typical in those carotenoids having keto group(s) in the conjugated system of the β-ionone ring (Fig. 9; marked with an arrow). Carotenoid 5 was identified as 2-hydroxyastaxanthin (see Fig. 2 and Example 13). From the results described so far, it was elucidated that the β-ionone ring-2-hydroxylase (gene product encoded by ORF11) is low (broad) in substrate specificity like other carotenoid biosynthesis enzymes.

[EXAMPLE 13] Identification of Pigments Converted by ORF11 (Known Substances)

A pUCBre-O11 and pACCAR25ΔcrtX-introduced *E. coli* clone was cultured in 2

L of 2xYT medium, and cells were harvested by centrifugation at 8,000 rpm for 10 min. The cells were suspended in STE buffer (see Example 3) and centrifuged at 8,000 rpm for 10 min to harvest the cells again. Acetone-methanol (1:1) (400 ml) was added to the cells and agitated for 1 hr. After filtration, the filtrate was vacuum-concentrated to thereby obtain 267 mg of extract, which was then separated by Silica Gel-60 (15 g) column chromatography. Carotenoids were eluted with 100 ml each of hexane-ethyl acetate solvents (8:2) (7:3) (6:4) and (1:1) in succession. As a result, three colored fractions were obtained. One was zeaxanthin. The remaining two fractions were identified by HPLC-PDA-MS analysis and ^{1}H-NMR analysis. For HPLC-PDA-MS analysis, Nano Space SI-2 (Shiseido) was used as a semi-micro HPLC system equipped with a PDA (photodiode array) detector. To this system, an ion trapping type mass spectrometer LCQ advantage system (ThermoQuest) was connected. As a column, a Develosil C30-UG-3 (1.0 mm i.d. x 150 mm) (Nomura Chemical) which is a C30 column was used. As a pre-column, Develosil C30-UG-S was used. Carotenoids were eluted at a flow rate of 1.0 ml/min with solvent A (96% methanol) for 12 min, then by a gradient from solvent A to solvent B (tert-methyl butyl ether (TMBE)) (B: 0-60%, from 12 min to 72 min), and finally eluted under that state from 72 min to 82 min. MS was detected by Atmospheric Pressure Chemical Ionization (APCI). ^{1}H-NMR was measured with INOVA750 system (Varian) in deuterated chloroform.

As a result of HPLC-PDA-MS analysis (retention time (RT) 13.48 min; λmax 449, 475 nm; m/z 601 $[M+H]^+$, 583 $[M+H-H_2O]^+$, 565$[M+H-2H_2O]^+$; and RT 17.75min; λmax 450, 476 nm; m/z 585 $[M+H]^+$, 567 $[M+H-H_2O]^+$) and ^{1}H-NMR analysis, the carotenoids present in the above-described two fractions were identified as nostoxanthin (2,2'-dihydroxyzeaxanthin) and caloxanthin (2-hydroxyzeaxanthin) (Buchecker, R., Liaaen-Jensen, S., Borch, G., Siegelman, H. W., "Carotenoids of blue-green algae. Part 9. Carotenoids of *Anacystis nidulans*, structures of caloxanthin and nostoxanthin", Phytochemistry 15, 1015-1018, 1976) (see Fig. 10).

Hereinbelow, the ^{1}H-NMR data (δ ppm; in parentheses are number of hydrogen atoms, multiplicity, and coupling constant) are shown. Nostoxanthin: 1.01 (6H, s), 1.14 (6H, s), 1.72 (6H, s), 1.98-1.99 (12H, s), 2.15 (2H, dd, J=17.4, 10.0Hz), 2.4 9(2H, dd, J=17.4, 6.7Hz), 3.33 (2H, d, J=10.0Hz), 3.84 (2H, dt, J=6.7, 10.0Hz), 6.0-6.7 (14H, m); Caloxanthin: 1.01 (3H, s), 1.08 (6H, s), 1.14 (3H, s), 1.49 (1H, t, J=12.0Hz), 1.72 (3H, s),

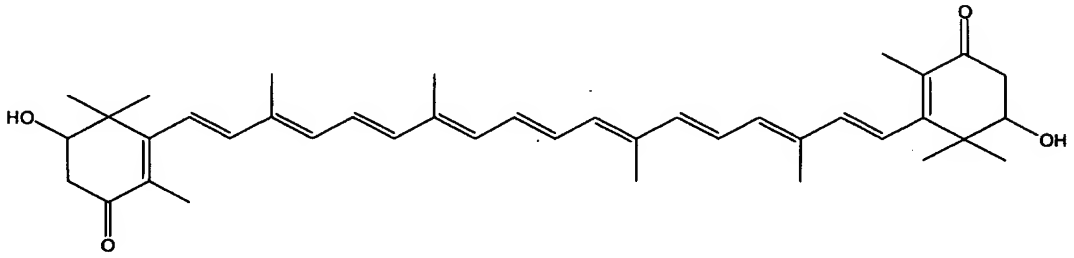
1.75 (3H, s), 1.80 (1H, m), 1.98-1.99 (12H, s), 2.05 (1H, dd, J=17.4, 10.5Hz), 2.15 (1H, dd, J=17.4, 10.0Hz), 2.40 (1H, dd, J=17.4, 6.3 Hz), 2.49 (1H, dd, J=17.4, 6.7Hz), 3.33 (1H, d, J=10.0 Hz), 3.84 (1H, dt, J=6.7, 10.0 Hz), 4.01 (1H, m), 6.0-6.7 (14H, m).

A pUCBre-O11 and pAC-Asta-introduced *E. coli* clone was cultured in 2 L of 2xYT medium, and cells were harvested by centrifugation at 8,000 rpm for 10 min. The cells were suspended in STE buffer (see Example 3) and centrifuged at 8,000 rpm for 10 min to harvest the cells again. Acetone-methanol (1:1) (400 ml) was added to the cells and agitated for 1 hr. After filtration, the filtrate was vacuum-concentrated to thereby obtain 27 mg of extract, which was then separated by Silica Gel-60 (15 g) column chromatography. Carotenoids were eluted with 100 ml each of hexane-ethyl acetate solvents (7:3) (6:4) and (1:1) in succession. As a result, three colored fractions were obtained. Two were astaxanthin and adonixanthin. The remaining one fraction was identified by HPLC-PDA-MS analysis (RT 11.98 min; λmax 473 nm; m/z 613 $[M+H]^+$) and ^{1}H-NMR analysis. As a result, the carotenoid was identified as 2-hydroxyastaxanthin (Non-Patent Document 2) (see Fig. 2). Hereinbelow, the ^{1}H-NMR data are shown. 1.22 (3H, s), 1.27 (3H, s), 1.30 (3H, s), 1.33 (3H, s), 1.82 (1H, m), 1.96 (6H, s), 1.98-2.01 (12H, s), 2.17 (1H, bm), 3.53 (1H, m), 4.19 (1H, m), 4.33 (1H, m), 6.2-6.7 (14H, m). Although 2,3,2',3'-tetrahydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxyastaxanthin) could not be confirmed this time, it is believed that this carotenoid can be obtained by, for example, appropriately selecting culture conditions or the like.

[EXAMPLE 14] Identification of Pigments Converted by ORF11 (Novel Substances)

A pUCBre-O11 and pAC-Cantha-introduced *E. coli* clone was cultured in 2 L of 2xYT medium, and cells were harvested by centrifugation at 8,000 rpm for 10 min. The cells were suspended in STE buffer (see Example 3) and centrifuged at 8,000 rpm for 10 min to harvest the cells again. Acetone-methanol (1:1) (400 ml) was added to the cells and agitated for 1 hr. After filtration, the filtrate was vacuum-concentrated to thereby obtain 85 mg of extract, which was then separated by Silica Gel-60 (15 g) column chromatography. Carotenoids were eluted with 100 ml each of hexane-ethyl acetate solvents (8:2) (7:3) and (1:1) in succession. As a result, three colored fractions were obtained. One was canthaxanthin. The remaining two fractions were identified by HPLC-PDA-MS analysis (RT 9.30 min; λmax 472 nm; m/z 597.2 $[M+H]^+$; and RT 17.62

min; λmax 474 nm; m/z 581.2 $[M+H]^+$), high resolution (HR) FABMS analysis, and ^1H and various two dimensional NMR analyses. As a result, these carotenoids were identified as 2,2'-dihydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxycanthaxanthin; novel compound (I)) and 2-hydroxy-β,β-carotene-4,4'-dione (2-hydroxycanthaxanthin), respectively (see Fig. 10).



(I)

Hereinafter, HRFABMS analysis data and ^1H-NMR data are shown. 2,2'-dihydroxy-β,β-carotene-4,4'-dione: HRFABMS (m/z, $[M]^+$), calculated: 596.3866 ($C_{40}H_{32}O_4$), measured: 596.3863, ^1H NMR (750 MHz, in deuterated chloroform, δ ppm),1.22 (6H, s), 1.26(6H, s), 1.89 (6H, s), 2.00-2.02 (12H, s), 2.62 (2H, dd, J=17.4, 9.0Hz), 2.80 (2H, dd, J=17.4, 4.5Hz), 3.90 (2H, dd, J=9.0, 4.5Hz), 6.2-6.7 (14H, m). 2-hydroxy-β,β-carotene-4,4'-dione: HRFABMS (m/z, $[M]^+$), calculated: 580.3916 ($C_{40}H_{32}O_3$), measured: 580.3900, ^1H NMR, 1.21 (6H, s), 1.22 (3H, s), 1.26 (3H, s), 1.85 (2H, t, J=7.0Hz), 1.89 (3H, s), 1.90 (3H, s), 2.00-2.02 (12H, s), 2.51 (2H, t), 2.62 (1H, dd, J=17.4, 9.0Hz), 2.8 (1H, dd, J=17.4, 4.5Hz), 3.90 (1H, dd, J=9.0, 4.5Hz), 6.2-6.7 (14H, m).

2,2'-Dihydroxy-β,β-carotene-4,4'-dione in which a hydroxyl group is introduced at both positions 2 and 2' of canthaxanthin is a novel compound which has not yet been found in nature, as described above. Further, 2-hydroxy-β,β-carotene-4,4'-dione in which a hydroxyl group is introduced at position 2 alone is only reported to have been isolated from a crustacean *Daphinia magna*, and this one produced in the present invention is the first 2-hydroxy-β,β-carotene-4,4'-dione produced by a microorganism (Partali, V., Olsen, Y., Foss, P., Liaaen-Jensen, L., "Carotenoids in food chain studies-I. Zooplankton (*Daphnia magna*) response to a unialgal (*Scenedesmus acutus*) carotenoid diet, to spinach, and to yeast diets supplemented with individual carotenoids", Comp. Biochem. Physiol.,

82B(4), 767-772, 1985; Foss, P., Partali, V., Olsen, Y., Borch, G., Liaaen-Jensen, S., "Animal carotnoids 29. New (2R)-2-hydroxy-4-keto-β-type carotenoids from *Daphnia magna* (Crustaceae)", Acta Chemica Scandinavica B40,157-162, 1986).

[EXAMPLE 15] Measurement of *in vitro* Anti-Peroxidation Activity

Although various carotenoids have a wide variety of physiological activities and effects, it is believed that activity to inhibit peroxidation of lipids in the living body caused by oxygen radicals is the basis for all activities/effects. Therefore, *in vitro* inhibitory effects of the two carotenoids synthesized in Example 14 on lipid peroxidation caused by free radicals were examined using rat brain homogenate. This assay was conducted basically according to the method of Kubo et al. (Kubo, K., Yoshitake, Y., Kumada, K., Shuto K., Nakamizo, N. "Radical scavenging action of flunarizine in rat brain *in vitro*". Arch. Int. Pharmacodyn. Ther. 272, 283-295, 1984). Briefly, 0.05 ml of methanol solution of a test sample, 0.1 ml of 1 mM ascorbic acid (final concentration 100 μM) and 0.05 ml of H_2O were added to 0.6 ml of 100 mM phosphate buffer (pH 7.4) and pre-incubated at 37°C for 5 min. Then, 0.2 ml of 2.5% (w/v) rat brain homogenate was added thereto to start reaction, followed by incubation at 37°C for 1 hr under shaking. One milliliter of a mixed solution containing 20% (w/v) trichloroacetic acid, 0.5% (w/v) 2-thiobarbituric acid and 0.2 N HCl was added to the above reaction solution to terminate the reaction. The resultant solution was boiled at 100 °C for 30 min for color development. After cooling, the solution was centrifuged at 3000 rpm for 5 min. Absorbance at 532 nm (A_{532}) of the resultant supernatant was measured. The concentration of test sample which is required to decrease the A_{532} of test sample addition group to one half of the A_{532} of test sample non-addition group was calculated as IC_{50}. This value was taken as lipid peroxidation inhibitory effect in rat brain homogenate. The results of this experiment are shown in Table 4. As is clear from Table 4, 2-hydroxy-β,β-carotene-4,4'-dione (2-hydroxycanthaxanthin) and 2,2'-dihydroxy-β,β-carotene-4,4'-dione (2,2'-dihydroxycanthaxanthin) have strong inhibitory effects on lipid peroxidation. Especially, the latter (novel substance) exhibited a strong inhibitory effect on lipid peroxidation.

Table 4

Inhibitory Effects of Carotenoids with 2-Hydroxyl Group and 4-Keto Group on Lipid Peroxidation in Rat Brain Homogenate

Carotenoid	IC_{50} (μM)
β-Carotene	>100
Canthaxanthin	50
2-Hydroxycanthaxanthin	8.1
2,2'-Dihydroxycanthaxanthin	4.8

The present specification encompasses the contents disclosed in the specifications and/or drawings of Japanese Patent Applications No. 2003-388165 and No. 2004-165919 based on which the present patent application claims priority. All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.